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Exogenous γ-aminobutyric acid mitigates drought-induced impairments in *Thymus daenensis* Celak by regulating physiological traits, antioxidant enzymes and essential oil constituents

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Abstract

Drought stress is one of the most significant environmental challenges, leading to various changes in the physiological processes of plants. Gamma-aminobutyric acid (GABA) is an essential biomolecule that plays a critical role in regulating growth and stress signaling. The current study aims to investigate the effects of GABA on antioxidant enzyme activity and the essential oil composition of *Thymus daenensis* Celak under different levels of water deficit stress. We examined three different levels of soil moisture (90%, 60%, and 30% of field capacity) alongside three GABA foliar treatments (0 mM, 25 mM, 50 mM, and 75 mM). The results showed that applying 25 mM GABA under severe stress conditions (at 30% of field capacity) significantly increased the activity levels of ascorbate peroxidase, guaiacol peroxidase, and superoxide dismutase enzymes by 95.5%, 78.45%, and 38%, respectively. Additionally, applying GABA at various levels during different water stress treatments led to significant improvements in the chlorophyll, carotenoid, and proline content in leaf tissues. Specifically, the application of 25, 50, and 75 mM GABA enhanced the proline content in *T. daenensis* by 53.9%, 26.8%, and 11%, respectively, compared to the control group (non-application of GABA). Essential oil analysis revealed the following ranges: thymol was present in the range of 38.07–45.18%, cymene in the range of 5.13–14.10%, caryophyllene in the range of 4.3–23.01%, and cineole in the range of 2–4.15%. The highest amount of thymol was obtained in the absence of GABA at 30% field capacity, while the greatest amount of cymene was also observed without GABA at 90% field capacity. Additionally, the maximum concentration of caryophyllene was found when 50 mM GABA was applied at 90% field capacity, and the maximum level of cineole was detected with 75 mM GABA at 90% field capacity. In conclusion, the exogenous application of GABA demonstrated favorable efficacy, particularly at a concentration of 25 mM. This treatment resulted in a significant enhancement of the plant's defense mechanisms and created favorable conditions that notably impacted the quality of the essential oil produced by *T. daenensis*.

Keywords Photosynthetic pigments, Thymol, Essential oil compounds, Enzymatic antioxidant activity, Proline

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Introduction

Thymus daenensis Celak is a perennial plant that belongs to the Lamiaceae family [66]. It is commonly used in beverages, as a food flavoring, and in herbal medicine [61]. The subsp. Daenensis of *T. daenensis* is believed to have originated in Iran and is found in the central and western regions, as well as the Zagros mountains [75, 85]. This plant is well-known for its essential oils, which contain compounds such as thymol, carvacrol, paracymene, beta-bisabolene, trypinen 4L, borneol, and spachulenol [73].

Drought stress significantly impairs the growth and development of crops [57, 10, 57, 51, 55, 56]. Moreover, drought stress induces a secondary oxidative stress response, which adversely affects physiological processes and photosynthetic pigments [3, 94]. This response prompts plants to produce various reactive oxygen species, such as superoxide and hydroxyl radicals, nitric oxide, and singlet oxygen, as a means of adapting to environmental challenges [7]. Plants have inherent defense mechanisms to counteract oxidative stress, which includes the production of osmolytes like soluble proteins, proline, and soluble carbohydrates. Additionally, plants synthesize antioxidants such as flavonoids, carotenoids, vitamins, and enzymes. These defense mechanisms involve the synthesis of several key antioxidants in response to abiotic stresses, including glutathione reductase (GTX), superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) [22, 41, 76]. The quality and quantity of medicinal plants largely depend on their secondary metabolites [36, 43, 78]. Essential oils in plants act as a defense mechanism against environmental stresses, including drought. These substances often undergo significant changes in response to such stresses [8]. It is important to note that variations in essential oils are considered a crucial defense mechanism regulated by occurrences of drought stress [8].

Today, various strategies are employed to mitigate the harmful effects of environmental stress on plants. One of the simplest and most effective methods involves the use of biomolecules that can activate resistance mechanisms, enhancing plant tolerance [83]. Gamma-aminobutyric acid (GABA) is a widely distributed compound in plants that plays a significant role in stress responses, signaling, and storage [32]. GABA acts as an essential signaling molecule that regulates plant growth and development, influences cell pH, affects osmotic potential, and provides defense against oxidative stress. It also helps plants adapt to both biotic and abiotic stresses [60, 79]. Research shows that the exogenous application of GABA enhances growth and performance in crops under drought stress, stimulates antioxidant defense mechanisms, and reduces oxidative damage [84]. Studies indicate that using GABA during drought stress not only promotes growth mechanisms but also significantly increases the activity of antioxidant enzymes such as superoxide dismutase, catalase, and proline [32].

Global warming and climate change have led to reduced rainfall and increased evapotranspiration, particularly in arid and semiarid regions. As a result, drought and heat stress have negatively impacted crop growth and production in these areas. There is limited information regarding the effectiveness of GABA (gamma-aminobutyric acid) application on the quality and quantity of essential oils in medicinal and aromatic plants, such as Thymus daenensis, under various biotic and abiotic stresses. The cultivation of *T. daenensis* is particularly significant in Iran due to its unique ecological, economic, cultural, and medicinal value. With water scarcity becoming increasingly critical, Iran needs to focus on crops that are suitable for its environmental conditions. Therefore, this experiment aims to investigate the effects of different levels of GABA application on the morphological and physiological characteristics, as well as the essential oil quantity and quality of *T. daenensis*, under varying levels of water deficit. We hypothesize that GABA application will alleviate the negative effects of drought stress, enhance antioxidant activity in the plants, and improve both the quantity and quality of essential oils in *T. daenensis* seedlings.

Materials and methods

Plant materials and growth conditions

This study aimed to examine how different levels of GABA affect the activity of antioxidant enzymes and the physiological traits of *T. daenensis* subsp. Daenensis under simulated water deficit stress conditions. The experiment took place in a controlled environment within the research greenhouse of Maragheh University. Thyme seeds (obtained from Pakan Bazr company, Isfahan, Iran) were initially sown in trays, and after four weeks from planting the seedlings were then transferred to paper pots before being transplanted into larger pots. The pots (5-L with a diameter of 21 cm × 25 cm height) were filled with soil with specific dimensions. The thyme plants were grown in a clay soil (Table 1), at 25 ± 2 °C temperature, 65% relative humidity (RH) and a daily photoperiod of 8h.

Plant treatments and application of water deficit stress

For this purpose, a factorial experiment was conducted based on the randomized complete block design at three different levels of soil water moisture (90, 60 and 30% of FC) and 4 levels of GABA foliar application [0 or non-application (control), 25, 50 and 75 mM] with three replications.

Table 1 Physico-chemical properties of soil used in this study

Soil Characteristics Measured in the Soil	Values	Soil Characteristics Measured in the Soil	Values
Soil Textures	Clay	Total Nitrogen (%)	0.11
Soil Clay Acidity	8.17	P availability (mg/kg)	6.38
(Desi Siemens per meter) Electrical Conductivity	0.36	K availability (mg/kg)	332
Calcium Carbonate equivalent	10.04	Zn availability (mg/kg)	0.31
Organic Carbon	0.25	Mn availability (mg/kg)	1.32
Saturation Moisture	41	Fe availability (mg/kg)	2.22

Table 2 Mean comparison of effect of different concentration of GABA on physiological traits of *Thymus daenensis* Celak under water deficit stress

Drought stress	Treatment	H_2O_2 (mmolg^{-1} FW)	MDA (nmolg^{-1} FW)	Proline (mg g^{-1} FW)	Chl a (nmol g^{-1} FW)	Chl b (nmol g^{-1} FW)	Carotenoid (mmol g^{-1} FW)
90% FC (no stress)	**Non application**	0.393c	6.2de	13.33f	0.917c	0.6867bc	0.5233 cd
	25 Mm GABA	0.3c	5.23e	14.23f	3.433a	1.8333a	0.73a
	50 Mm GABA	0.33c	5.53e	13.73f	1.177c	0.8633b	0.61abc
	75 Mm GABA	0.37c	5.8de	13.8f	1.157c	0.81b	0.5867abc
60% FC	**Non application**	1.6b	13.3b	18.87e	0.653 cd	0.4867bcd	0.51 cd
	25 Mm GABA	0.57c	6.8de	38.63a	1.933b	1.6667a	0.71ab
	50 Mm GABA	0.647c	8.47 cd	27.17c	0.867c	0.7467bc	0.5867abc
	75 Mm GABA	0.733c	9.6c	23.17d	0.703 cd	0.6667bc	0.5667bc
30% FC	**Non application**	2.367a	18.43a	22.03d	0.253d	0.1833d	0.3233e
	25 Mm GABA	1.9ab	16.77a	30.6b	1.933b	0.7867b	0.6533abc
	50 Mm GABA	2.033ab	17.33a	27.9bc	0.64 cd	0.3767 cd	0.4de
	75 Mm GABA	2.143ab	17.63a	23.23d	0.613 cd	0.2533d	0.3433e

In each column there is no significant difference between means with same letters by LSD ($P \leq 0.05$)

For measuring the soil water content, a Time-Domain Reflectometry (TDR) probe (Model TRIME-FM, England) was used. GABA foliar spraying was performed two times (1 day and 7 day) after application of water deficits. Control plants were sprayed using distilled water.

Extraction and assay of antioxidative enzymes

In flowering stage, the fresh tissues (0.5 g fresh weight) were homogenized in 2 mL of 100 mM potassium phosphate buffer, pH 7 containing 1 mM of EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). The subsequent centrifugation was conducted at a speed of 15,000 times the force of gravity at a temperature of 4 °C for a duration of 15 min, following the procedure described by Sairam et al. [72]. The extract was then centrifuged at 4 C for 15 min at 12.000 g in a cooled centrifuge. This supernatant was used to measure the activities of superoxide dismutase (SOD), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT).

Activity of SOD was assayed by using the photochemical nitro blue tetrazolium (NBT) method. The assay was performed in terms of SOD's ability to inhibit reduction of NBT to form formazan by superoxide radical as described by Beauchamp and Fridovich [18]. Activity of POD was determined at 25 °C with guaiacol [19]. Activity of APX was measured by following the rate of hydrogen peroxide-dependent oxidation of ascorbic acid [59]. The CAT activity was strictly determined at 25 °C using UV–vis spectrophotometry [96]. The reaction mixture was made up of 1.5 mL of 0.05 M sodium phosphate buffer (pH = 7.8), 1 mL of deionized water, 0.3 mL of 0.1 M H_2O_2, and 0.2 mL of enzyme extract. The catalase activity was precisely calculated by monitoring the absorbance at 240 nm due to H_2O_2 consumption and was presented as unit mg^{-1} protein.

Measurement of Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide content was measured based on Chen et al. [23]. Shoots were frozen with liquid nitrogen and grounded with 5 ml 0.1% (w/v) trichloroacetic acid. The mixture was centrifuged at 15.000×g for 15 min at 4 °C. Supernatant (0.5 ml) was aliquoted and 1 ml 1 M KI and 0.5 ml 10 mM K_2HPO_4 buffer (pH 7.0) was added. Reaction mixture was subjected to dark conditions for 60 min. The absorbance values of the samples were read at 390 nm. H_2O_2 amount was calculated with a standard curve.

Estimation of lipid peroxidation

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content of shoots as described by Heath and Packer [34] through a colorimetric method. Shoot samples were homogenized in 2 ml of 0.1% trichloroacetic acid (TCA) and centrifuged. Then, 0.5 ml of supernatant was mixed with 2 ml of 20% TCA containing 0.5% thiobarbituric acid. The mixture was incubated at 95 °C for 30 min. The samples were centrifuged at 10,000 *g* for 10 min. The absorbance of the supernatant was read at 532 and 600 nm. The amount of MDA was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

Chlorophyll content

In order to quantified the content of chlorophyll a (Cha), b (Chb) and carotenoid, 0.5 gr of fresh leaves digested in liquid nitrogen and mixed with 10 mL of 80% ace-tone. Samples centrifuged at 10,000 rpm and supernatant after 10 min transferred to a new tube and the absorbance was read at 645, 663, and 470 nm spectrophotometrically (UV-1800, Shimadzu, Japan). After that, content of photosynthetic pigments calculated by following equations [12]:

$$\text{Cha} = [(12.7 \times \text{Abs}_{663}) - (2.69 \times \text{Abs}_{645})]$$

$$\text{Chb} = [(21.5 \times \text{Abs}_{645}) - (5.1 \times \text{Abs}_{663})]$$

$$\text{Carotenoid} = [(1000 \times \text{Abs470}) - (1.82 \times \text{Cha} - 85.02\text{Chb}/198)]$$

Malondialdehyde content

For the determination of Malondialdehyde (MDA), firstly, 0.1 g of fresh leaves sample extracted using 1 mL 0.1% TCA (trichloroacetic acid) and centrifuged at 12,000 rpm. After 15 min, the supernatant mixed with 4 mL of reaction mixture including 20% TCA and 0.67% 2-thiobarbituric acid (TBA). After that, the sample heated in water bath at 95 °C for 15 min and then quickly cooled down ice bath for 10 min and centrifuged at 10,000 rpm for 5 min at 4 0C. Finally, absorbance was recorded at 532 and 600 nm spec-trophotometrically (UV-1800, Shimadzu, Japan) and the MDA concentration represent as nmol g^{-1} fresh weight [34, 35].

Essential oil extraction and analysis

Essential oils were extracted using a Clevenger type apparatus for three hours. Then dried over anhydrous sodium sulphate and kipped in the dark vials until analysis. For Essential oil components analysis, a GC device connected to MS (GC-MA) Agilent 5977A model was used. The column conditions were as follow: an HP-MS Column, 5% phenylmethyl polysiloxane, 30 m long, 0.25 mm i.d., 0.25 µm film thickness). The oven temperature program, was programed as follow: 60 °C for 5 min, then the temperature increased to 240 °C at a rate of 3 °C/min then kept for 10 min. The carrier gas was Helium with a flow rate of 1 ml/min. The ionization voltage was 70 eV, transfer line temperatures sets at 250 °C. The same instrument with HP5 column was used for GC-FID analysis. The identification and quantification of components was done according to the procedure explained by Akbarzadeh et al. [3].

Statistical analysis

The normality and homoscedasticity of data regarding the studied traits were verified using the Kolmogorov–Smirnov and Levene test, respectively. Analysis of variance appropriate to the experimental design was conducted, using SPSS and SAS software. Means of each trait were compared according to Duncan multiple range test at $P \leqslant 0.05$. Excel software was used to draw figures.

Results and discussion

Ascorbate peroxidase

The comparison of mean ascorbate peroxidase enzyme activity revealed that applying 25 mM GABA under 60% FC stress significantly enhanced enzyme activity compared to other treatments. However, this enhancement was not observed with the 50 mM GABA treatment under the same water conditions. The study indicated a notable reduction in enzyme activity due to both water deficit stress and the application of high levels of GABA. Data presented in Fig. 1 showed that exposure to water deficit stress up to 60% of FC increased enzyme activity, but further exacerbation of stress led to a decrease in activity. According to Ahmadian et al. [6], increased antioxidant enzyme activity is a crucial mechanism for plants to mitigate abiotic and biotic stresses. Under stress conditions, ascorbate peroxidase enzyme levels tend to rise. Additionally, Iqbal et al. [37] observed a significant

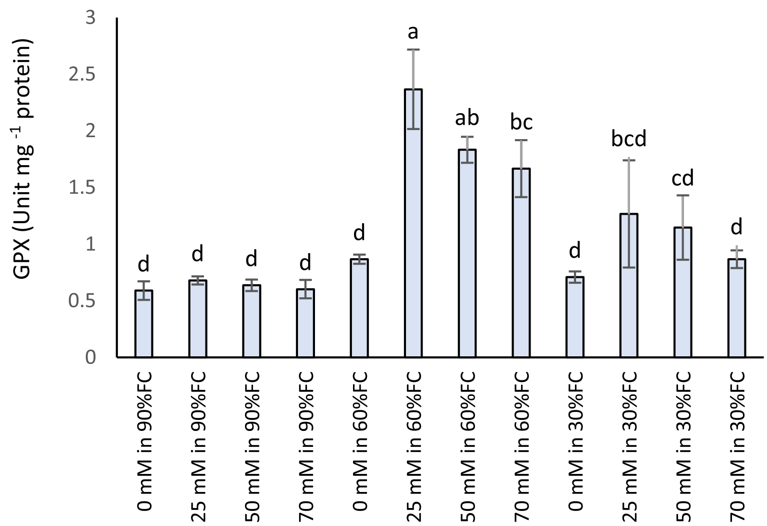

Fig. 1 The interaction of water deficit stress and GABA foliar spraying on the level of ascorbate peroxidase enzyme activity. Means followed by the same letters within the same column for each factor indicate no significant difference ($p < 0.05$). The means separation is done using Duncan's new multiple range test (DMRT) analysis

increase in ascorbate peroxidase activity in response to water stress induced by polyethylene glycol, compared to control conditions. Similarly, heightened transcription and enzyme activity were noted following the exogenous application of GABA [33]. Kalhor et al. [40] examined the impact of exogenous GABA on lettuce plants under stressful conditions and reported a significant increase in ascorbate peroxidase activity. Furthermore, Ghahremani et al. [31] investigated the effects of GABA on perennial ryegrass subjected to drought stress and found that applying a concentration of 70 mM GABA resulted in reduced ascorbate peroxidase activity compared to 50 mM GABA. This suggests a change in how GABA influences enzyme functionality in response to drought stress.

Catalase

The comparison of catalase enzyme activity showed variability in response to different stress levels. Initially, the enzyme activity increased but then decreased as stress levels rose. Notably, when 25 mM GABA was applied at 60% FC, there was a significant increase in catalase enzyme activity. The results indicated a reduction in enzymatic activity with higher concentrations of GABA, particularly under stress conditions (Fig. 2). Catalase is an important antioxidant enzyme that plays a critical role in removing reactive oxygen species (ROS) during periods of heightened stress [53]. Previous research by Bahari et al. [14] suggested a positive correlation between stress intensity and catalase activity, finding that as field capacity decreased from 70 to 30%, catalase activity increased. However, other reports indicated that as stress levels increase, the activity of antioxidant enzymes like catalase can decrease, leading to significant cellular damage. Rezayian et al. [69] highlighted that elevated stress levels, particularly those caused by higher concentrations of polyethylene glycol, resulted in reduced activity and expression of the catalase enzyme gene. In contrast, findings by Li et al. [46] emphasized the role of GABA in upregulating gene expression related to antioxidant enzymes, such as catalase, during drought-induced stress. Abd El-Gawad et al. [2] revealed that applying varying amounts of GABA during drought conditions led to increased catalase enzyme activity compared to non-stressed situations. They observed a notable increase in catalase activity with rising GABA concentrations (ranging from 0.5 to 2 mM), indicating GABA's role in stimulating the production of antioxidant genes. Seifikalhor et al. [77] noted a reduction in catalase enzyme activity in untreated plants subjected to drought stress. However, exogenous application of GABA resulted in a substantial enhancement of the enzyme's activity, especially when plants were exposed to elevated stress levels (20% and 40% FC). At 20% of FC, catalase activity increased with the application of 25 µM GABA but decreased when the concentration was raised to 50 µM (Fig. 3).

Guaiacol peroxidase

The data indicates that the guaiacol peroxidase enzyme responds in a complex manner to water deficit stress (Fig. 3). Initially, a slight, statistically insignificant increase in enzyme activity was observed with incremental stress; however, this was followed by a decrease at higher stress levels. Notably, the application of GABA

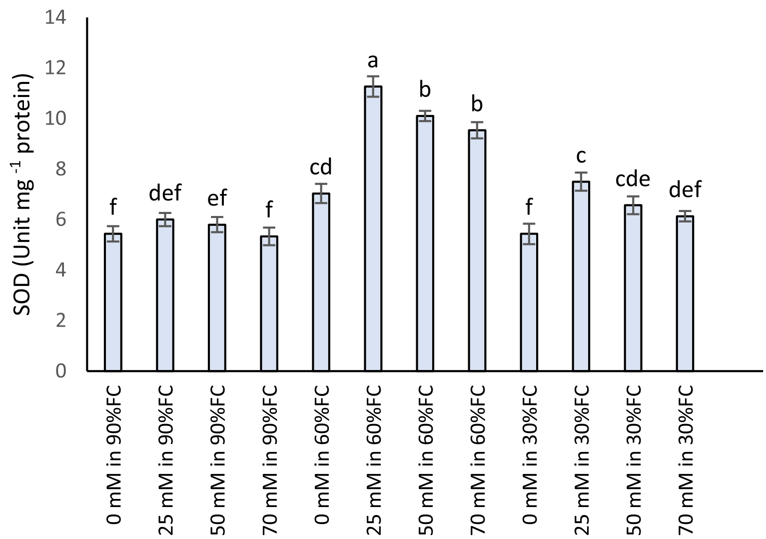

Fig. 2 The interaction of water deficit stress and GABA foliar spraying on the level of catalase enzyme activity. Means followed by the same letters within the same column for each factor indicate no significant difference ($p < 0.05$). The means separation is done using Duncan's new multiple range test (DMRT) analysis

resulted in a significant increase in guaiacol peroxidase activity. The enzyme activity reached its peak under water deficit stress conditions when treated with 25 mM GABA at a 60% field capacity (FC) intensity level. Nevertheless, increasing the GABA concentration from 25 to 70 mM under stress conditions corresponding to 60% and 30% of FC led to a reduction in guaiacol peroxidase activity. Plants experiencing stress tend to generate more reactive oxygen species (ROS), which heightens their reliance on antioxidant enzyme defenses [6]. Guaiacol peroxidase is a crucial antioxidant enzyme that often shows significant increases in activity under drought stress in various plant species [13, 58]. Research has demonstrated notable enhancements in guaiacol peroxidase activity at heightened stress levels, particularly at varying intensities of field capacity. Cheng et al. [24] found that applying GABA during stress increases the expression of several antioxidant enzymes, particularly guaiacol peroxidase. In a study involving white clover (*Trifolium repens* L.), the application of GABA under stressful conditions resulted in a substantial increase in enzyme transcription, leading to enhanced enzyme activity. These findings align with the results reported by Vijayakumari and Puthur [88] in black pepper (*Piper nigrum* L.) plants, where the application of GABA under stress increased guaiacol peroxidase activity beyond control levels. Overall, this study highlights the complex relationship between guaiacol peroxidase and its modulation in response to stress and exogenous GABA application, which is consistent with patterns observed across various plant species.

Superoxide dismutase

The results demonstrate a positive correlation between water deficit stress and the activity of the superoxide dismutase enzyme. Specifically, as water deficit stress increases, there is an initial rise in the enzyme's activity. However, as the stress intensity escalates from 60 to 30% of FC, there is a subsequent decrease in the enzyme's activity levels. Notably, the application of 25 mM GABA at 60% FC resulted in the highest activity of the superoxide dismutase enzyme. Additionally, as the concentration of GABA applied increased, there was a slight decline in the enzyme's activity, as illustrated in Fig. 4. Maintaining a balance between the generation of ROS and the protective antioxidant defense system is crucial for plant survival under unfavorable conditions. Superoxide dismutase serves as the primary line of defense [71], facilitating the enzymatic conversion of superoxide to hydrogen peroxide. Following this, several enzymes, including catalase, ascorbate peroxidase, and guaiacol peroxidase, convert hydrogen peroxide into harmless water and oxygen [28]. Rahimi et al. [65] investigated the impact of drought stress on *Thymus vulgaris* L. and observed a notable increase in superoxide dismutase activity during periods of stress. Similarly, a study by Sheteiwy et al. [80] showed that the external application of GABA to rice plants under stressful conditions led to an upregulation of superoxide dismutase gene expression. Notably, the application of exogenous GABA during saline-alkaline stress conditions correlated positively with increased superoxide dismutase activity in *Cucumis*

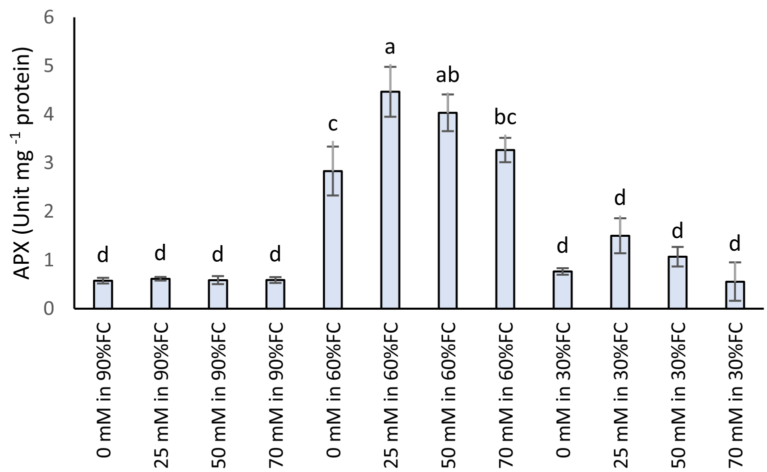

Fig. 3 The interaction of water deficit stress and GABA foliar spraying on the level of guaiacol peroxidase activity. Means followed by the same letters within the same column for each factor indicate no significant difference ($p < 0.05$). The means separation is done using Duncan's new multiple range test (DMRT) analysis

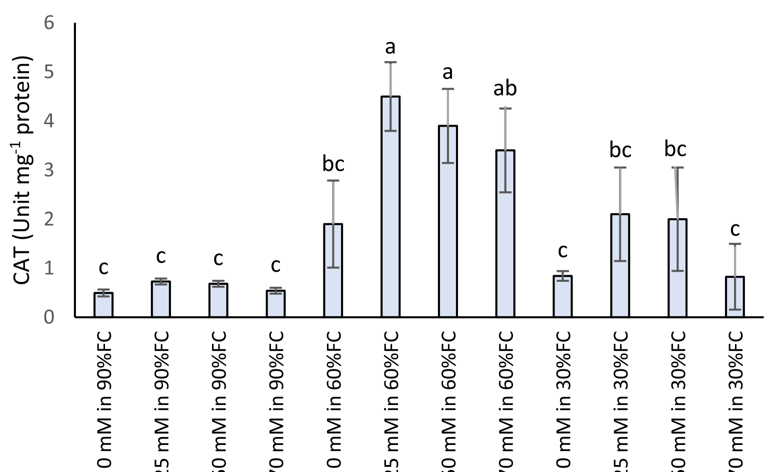

Fig. 4 The interaction of water deficit stress and GABA foliar spraying on the level of superoxide dismutase enzyme activity. Means followed by the same letters within the same column for each factor indicate no significant difference ($p < 0.05$). The means separation is done using Duncan's new multiple range test (DMRT) analysis

melon L. plants, as reported by Jin et al. [39]. These findings are consistent with recent studies that collectively highlight the significant impact of GABA application on enhancing superoxide dismutase activity across various plant species exposed to different stressors.

Hydrogen peroxide

The data indicate that increased concentrations of hydrogen peroxide occur in response to heightened water deficit stress. However, the application of 25 mM GABA results in a significant reduction in hydrogen peroxide production, particularly when the field capacity level reaches 60%. This decrease is noteworthy compared to the situation without GABA application (Table 2). Plants generate higher levels of reactive oxygen species (ROS) when faced with adverse environmental conditions. Research has shown that applying GABA during abiotic stress leads to a considerable reduction in ROS levels within plant tissues [44]. The activation of glutamate decarboxylase (GAD) under stress conditions helps regulate GABA through calcium, thereby enhancing the activity of the GABA-shunt. This process contributes to an increase in the photosynthetic rate and capacity, which in turn reduces ROS accumulation [30, 38, 47]. Consequently, treated plants exhibit improved membrane integrity [5]. Furthermore, utilizing GABA, particularly during stressful periods, activates genes responsible for producing antioxidant enzymes and boosts their activity, leading to lower hydrogen peroxide levels. GABA application has been shown to alleviate oxidative stress by improving carbon fixation, enhancing osmolyte production, and regulating leaf water status, as demonstrated by Wang et al. [90]. Additionally, Shi et al. [81] highlighted the significant role of GABA signaling in suppressing the expression of critical genes related to hydrogen peroxide production. Several studies, including research by Tang et al. [87], emphasize the effectiveness of applying GABA during drought-induced stress, as it helps reduce hydrogen peroxide levels by promoting the activity of antioxidant enzymes.

Malondialdehyde (MDA)

The study revealed that the highest concentrations of MDA were detected at 30% field capacity (FC), as shown in Table 2. Notably, applying GABA at lower concentrations and at 60% FC resulted in a significant reduction in MDA levels. The lowest MDA content was observed under non-stress conditions, at full FC, and with the application of 25 mM of GABA. MDA accumulation indicates membrane damage and lipid peroxidation caused by excessive ROS production, particularly under stress conditions (Sachdev et al., 1998). The findings indicated that the exogenous application of GABA decreased MDA levels in thyme seedlings, suggesting that GABA effectively mitigates the damage caused by drought stress to the cell membrane system. Additionally, GABA application increased the activity of antioxidant enzymes and the expression of genes related to these enzymes, such as SOD, CAT, APX, and POD, thereby reducing the negative effects of ROS and lowering MDA levels in plant cells. Ebrahimian et al. [27] demonstrated a significant increase in MDA levels in response to increased drought stress. Furthermore, GABA application under stress conditions has shown potential to suppress MDA production during lipid peroxidation [25]. Liu et al. [47] provided additional evidence supporting GABA's ability to alleviate stress through its inhibitory effects on various ROS. Existing data also suggest that GABA acts as a signaling molecule, inhibiting ROS and regulating the activity of antioxidant enzymes [16, 67]. Moreover, GABA application has been shown to enhance antioxidant enzyme activity, leading to a reduction in MDA levels [91]. The presence of amino acids in leaves may also contribute to protecting protein polymers within cells, further reducing MDA levels. GABA's exogenous application has demonstrated significant protective effects on tomato (*Solanum lycopersicum* L.) seedlings by alleviating oxidative damage and improving the plants' stress tolerance. Yong et al. [93] reported a notable increase in MDA levels due to drought stress. However, GABA treatment positively influenced membrane stability under such conditions. Additionally, internal GABA accumulation occurs during various stresses, including drought [21, 66], and the increase of internal GABA levels with external GABA application under stress has been confirmed [50]. It is crucial to note, however, that excessive GABA concentrations can result in toxicity, which is characterized by decreased antioxidant enzyme activity, increased ROS levels, and elevated MDA content, particularly under high-stress conditions (Table 2).

Chlorophyll a and b

The mean comparisons indicated a significant difference in chlorophyll content among the treatments (Table 2). Chlorophyll a and b levels showed a marked decrease as stress intensity increased, especially under severe drought conditions (Table 2). Conversely, applying exogenous GABA, particularly at a concentration of 25 mM, resulted in an increase in chlorophyll a and b levels. However, higher dosages of GABA were associated with a gradual decrease in pigment levels. The reduction in leaf chlorophyll content in response to stress conditions, such as drought, is a natural phenomenon [26]. Nitrogen is a crucial element in the structure of chlorophyll molecules. Under stressful conditions, the oversynthesis of proline disrupts nitrogen metabolism, which is linked to a decrease in chlorophyll content [4]. Water deficit negatively affects the light reactions of photosynthesis, leading to oxidative stress and consequent damage to both photosynthetic systems and pigments [29]. The activity of the chlorophyllase enzyme increases under stress, causing the breakdown of chlorophyll [62], which results in lower concentrations of chlorophyll a and b. The observed increase in chlorophyll levels with GABA application under stress conditions aligns with plant mechanisms designed to alleviate stress and protect photosynthetic pigments through the activation of the antioxidant system [48]. This suggests that applying GABA during stressful conditions may enhance the activity of antioxidant enzymes. The application of GABA has been linked to increased chlorophyll content [1], which is believed to optimize light-harvesting efficiency and regulate photosynthetic capacity. Similarly, applying GABA during water deficit conditions leads to an increase in leaf chlorophyll content due to elevated cell turgor pressure in treated plants compared to controls. Furthermore, the study emphasizes GABA's protective effect on the cell membrane [17].

Carotenoids

The research findings indicate that carotenoid concentrations significantly decrease in response to high stress levels. However, applying GABA at a concentration of 25 mM results in a notable increase in carotenoid levels under varying degrees of water deficit stress (see Table 2). Drought conditions can lead to the production of reactive oxygen species (ROS) in chloroplasts, which can damage chlorophyll molecules and chloroplast membranes [52]. Carotenoids are essential for protecting chloroplast membranes and inhibiting the photooxidation of chlorophyll during drought stress [86]. Moreover, GABA not only accelerates the biosynthesis of polyamines and their precursors but also prevents their decomposition, thereby enhancing carotenoid content [89]. The external application of GABA has also been shown to help maintain the structural and functional integrity of Photosystem II under stress-induced conditions [92]. Additionally, studies have revealed that GABA application under low light stress significantly increases carotenoid levels in pepper (*Capsicum annuum* L.) plants, demonstrating its potential to counteract the negative effects of stress on carotenoid content [45]. Overall, various studies suggest that the external application of GABA leads to significant increases in carotenoid concentrations, indicating its potential role as a regulator of carotenoid dynamics in stressful environments and its potential application for enhancing stress resilience in plants [9, 88].

Proline

Proline is well-known for its role as a protective agent for cytosolic enzymes, including carboxylase, as well as cellular structures. It is regarded as the most abundant and suitable osmolyte in response to stressful conditions [11, 42]. This investigation highlights the significant effect of varying levels of water deficit stress on proline concentration, showing a marked increase with higher stress intensity (Table 2). The glutamate pathway is the most common route for proline synthesis in plants, especially during stress when the conversion of glutamate to proline is elevated. Consequently, proline levels rise substantially in drought-induced stress situations. Furthermore, proline degradation is reduced under low water potential conditions, leading to increased overall proline levels [74]. Notably, the application of GABA has a considerable impact on proline synthesis during stressful conditions (Table 2). In common beans (*Phaseolus vulgaris* L.) subjected to normal irrigation, GABA application resulted in minimal changes in osmolyte concentrations. However, at a concentration of 25 mM, GABA effectively promoted osmotic tolerance under water deficit stress. Specifically, the application of 25, 50, and 75 mM of GABA enhanced the proline content in *T. daenensis* by 53.9%, 26.8%, and 11%, respectively, compared to the control (no GABA application). The accumulation of proline during water stress has been attributed to the favorable modulation of proline metabolism triggered by GABA application [2]. This increase in osmotolerant metabolites such as proline decreases osmotic potential and maintains turgor pressure in plant cells, thereby reducing the negative effects of reactive oxygen species (ROS) and lipid peroxidation, while enhancing membrane stability. Research conducted on white clover plants supports these observations, indicating that GABA application during drought stress promotes the accumulation of internal GABA and induces a favorable up-regulation of proline metabolism. Additionally, it has been noted that GABA application during drought stress increases the activity of P5CS, a crucial enzyme in proline production via the glutamate pathway [63, 93]. This study underscores the complex interaction among GABA, proline metabolism, and stress response pathways, offering valuable insights into GABA's potential role in regulating plant stress resilience through osmotic control.

Essential oil compounds

The composition of essential oil components in the aerial parts of the thyme plant was analyzed using Gas Chromatography-Mass Spectrometry (GC–MS). The essential oil content ranged from 95.77% to 99.98%, with thymol being the dominant component, constituting between 45.93% and 48.03% of the total (Table 3). Other notable components included p-cymene (10.03% to 11.75%), caryophyllene (E) (2.02% to 4%), cineole (1.03% to 4.11%), borneol (2.64% to 3.97%), γ-terpinene (1.85% to 3.79%), and carvacrol (2.07% to 3.88%). These results underscore the abundance of various components in Denai thyme essential oil. When the plants experienced water deficit stress, especially at 30% of field capacity (FC), the concentrations of key components such as cymene (11.75%), terpinene (3.79%), borneol (3.97%), thymol (48.03%), and carvacrol (3.88%) were notably higher. However, applying GABA at 30% of FC led to a decrease in these concentrations. In treatments without GABA application, the highest levels of cineole (4.11%) and phellandrene (5.98%) were recorded at 90% and 60% FC, respectively. Interestingly, conditions without water stress and without GABA treatment resulted in the lowest thymol level, at 45.93%. These observations indicate that water deficit stress tends to increase the levels of key components in Denai thyme. However, the application of GABA appears to counteract this effect, helping to mitigate the impact of water scarcity on the composition of essential oils. It is also important to note that the quantity and quality of essential oils in medicinal plants are influenced by ecological, genetic, geographic factors, and environmental pressures [95].

Table 3 Identified compounds of thyme and the percentage of its compounds under the influence of drought stress and GABA foliar application

				A1				A2				A3			
				B1	B2	B3	B4	B1	B2	B3	B4	B1	B2	B3	B4
Row	Essential oil components	RI	RI LIT												
1	α-Thujene	922.5	925	0.58	0.54	0.55	0.57	0.63	0.59	0.67	0.65	0.77	0.49	0.67	0.79
2	α-Pinene	928.7	932	0.83	0.8	0.85	0.83	0.97	0.92	0.93	0.91	1.03	0.92	1.03	1.29
3	Camphene	942.4	947	0.91	0.91	0.9	0.94	0.95	0.93	0.94	0.91	0.82	0.91	0.94	0.95
4	3-Octanone	982.2	979	0.09	0.53	0.04	0.55	0.47	0.39	0.4	0.42	0.73	0.51	0.67	0.71
5	Myrcene	987.4	988	0.63	0.61	0.6	0.63	0.66	0.55	0.59	0.51	0.87	0.71	0.84	0.93
6	α-Phellandrene	1000.6	1003	5.25	5.17	5.19	5.23	5.98	5.79	5.82	5.91	3.1	5.78	5.02	5.1
7	α-Terpinene	1012.3	1014	0.21	0.12	0.19	0.52	0.41	0.74	0.37	0.36	0.81	0.68	0.7	0.77
8	o-Cymene	1020.4	1022	10.5	10.9	10.07	10.41	10.31	11.03	10.03	10.11	11.75	10.07	10.2	11.09
9	1,8-Cineole	1025.8	1026	4.11	2.98	1.03	2.15	1.91	1.95	1.95	1.92	2.21	2	2	2.17
10	γ-Terpinene	1053.9	1054	2.8	3.62	3.75	2.81	2.72	3.03	1.85	2.07	3.79	1.98	2.76	2.73
11	Cis-Sabinene hydrate	1061.4	1065	0.47	0.42	0.49	0.45	0.52	0.55	0.51	0.55	0.73	0.6	0.67	0.69
12	trans-Sabinene hydrate	1092.8	1098	0	0	0	0	0.21	0.15	0.19	0.23	0.25	0.18	0.2	0.2
13	Linalool	1096.3	1096	0.43	0.54	0.47	0.44	0.49	0.42	0.49	0.41	0.57	0.43	0.49	0.54
14	Camphor	1141.5	1144	2.93	2.79	2.81	2.95	2.96	2.3	2.31	2.47	2.09	2.01	2.75	2.64
15	Borneol	1159.3	1166	3.42	2.71	3.42	3.39	3.21	2.94	3	2.97	3.97	2.64	3.36	3.01
16	cis-Dihydro carvone	1193.7	1195	0	0	0	0	0.24	0.72	0.21	0.24	0.3	0.21	0.25	0.22
17	Thymol, methyl ether	1230.6	1233	1.12	0.95	1.08	1.06	1.13	1.07	1.09	1.11	1.8	1.32	1.95	1.85
18	Carvacrol, methyl ether	1239.4	1243	2.9	2.5	2.91	2.78	2.3	2.18	2.9	3.1	2.11	3.61	3.72	2.02
19	Thymoquinone	1246.9	1250	3.07	2.93	3.14	2.04	2.12	2.02	2.1	1.98	2.2	2.07	2.18	2.11
20	Thymol	1294.1	1289	45.93	46.07	47.71	47.4	48	47.02	48	48	48.03	47.77	47.29	46.7
21	Carvacrol	1299.3	1299	2.86	2.07	3.1	3.48	3.65	2.52	3.19	3	3.88	2.9	3.32	3.87
22	(E)-Caryophyl-lene	1411.9	1419	4	2.9	2.23	2.91	2.96	3.01	2.95	2.9	3.15	4	3.05	2.02
23	Aromaden-drene	1431	1438	0	0	0	0	0.09	0	0.04	0.06	0.37	0.19	0.24	0.28
24	α-Humulene	1445.6	1455	0	0	0	0	0.08	0.03	0.03	0.06	0.27	0.13	0.17	0.21
25	Viridiflorene	1487.6	1498	0	0	0	0	0	0	0	0	0.08	0	0	0.03
26	β-Bisabolene	1502.9	1507	1.19	2.9	2.21	2.14	2.49	1.31	1.37	1.35	1.05	1.44	1.59	2.33

Table 3 (continued)

				A1				A2				A3		
				B1	B2	B3	B4	B1	B2	B3	B4	B1	B2	B3	B4
27	cis-α-Bisabolene	1537.7	1539	2.15	1.99	2.09	2.09	1.71	1.69	1.72	1.77	1.23	1.69	1.75	1.8
28	Caryophyllene oxide	1574.1	1583	3.04	2.87	2.99	2.95	2.17	2.77	2.12	2.08	1.1	2.11	2.17	2.09
	Total Identified (%)	-	-	99.42	97.82	97.82	98.72	99.34	96.62	95.77	96.05	99.06	97.35	99.98	99.14

In each column there is no significant difference between means with the same letters by LSD

A1, A2 and A3 indicated three different levels of soil water moisture (90, 60 and 30% of FC); B1, B2, B3 and B4 indicated 4 levels of GABA exogenous application [0 or non-application (control), 25, 50 and 75 mM]

Research on both wild and cultivated Denai thyme species provides compelling evidence supporting this assertion, highlighting significant differences in oil composition. Pirbalouti et al. [64] identified thymol, carvacrol, γ-terpinene, and p-cymene as the main components, with their respective percentages ranging from 39.3% to 70.3%, 4% to 24.8%, 3.9% to 10.4%, and 4.8% to 8.6%. According to Lubbe and Verpoorte [49], the variability in thymol and carvacrol levels in Denai thyme is closely linked to agronomic and environmental factors, particularly the effects of drought stress. Our findings align with previous reports indicating an increase in the production and percentage of thyme essential oil in response to drought conditions. Similarly, Bistgani et al. [20] highlight the positive effect of drought on essential oil accumulation. During periods of drought stress, reduced leaf surface area leads to an increase in the density of oil glands, which stimulates essential oil production [82]. Other factors contributing to enhanced essential oil production under drought include the reduced allocation of carbon to plant growth and the establishment of a balance between defense mechanisms (essential oil production) and plant growth [15], 54], (Pirbaluti et al., 2013).

The investigation into how plants can help reduce oxidative stress caused by drought is an important area of research. Under normal conditions, photosynthesis is limited by the concentration of atmospheric carbon dioxide (CO_2). When conditions are optimal, the production of NADPH, H^+, and ATP exceeds the requirements of the Calvin cycle, which helps stabilize and regenerate carbon dioxide. This process reduces $NADP^+$ levels and decreases the $NADP^+$/NADPH, H^+ ratio, leading to a decline in electron flow through the electron transport chain and an increase in ROS production. However, during drought stress, the closure of stomata limits the intake of carbon dioxide, worsening ROS production. To counter this and reduce ROS levels, medicinal plants work to regenerate the carbon components used in their metabolic structures. One of the methods they employ is the production of terpenes. Additionally, by introducing diversity in various functional groups, these plants can utilize more NADPH and H^+, which helps decrease ROS production. It is important to note that the synthesis of terpenes consumes NADPH, H+, and ATP. This consumption can hinder the transfer of electrons to oxygen molecules, ultimately reducing ROS production by adjusting the $NADP^+$/NADPH, H^+ ratio [78].

The study results indicated that the essential oil compounds in thyme are enhanced by the application of GABA. Specifically, GABA positively affects the content of photosynthetic pigments, such as chlorophylls a and b. The increased rate of photosynthesis, resulting from the higher pigment content, improves nutrient accessibility and helps modulate the negative effects of drought stress, which in turn boosts carbohydrate productivity. This boost supports cell growth and the production of essential oil-secreting glands, ultimately leading to an increase in essential oil content in medicinal and aromatic plants like thyme. Research has focused on the impact of GABA on the concentration of essential oils. According to Abd El-Gawad et al. [2], under non-stress conditions, GABA facilitates the uptake of essential mineral elements, including nitrogen, phosphorus, iron, zinc, calcium, and potassium. This increased mineral absorption is associated with a rise in essential oil proportions [3, 68, 70]. Under salt stress, GABA acts as a signaling molecule, influencing the expression of important enzyme genes and promoting the synthesis of secondary metabolites in soybean sprouts [97]. However, the relationship between GABA and essential oil synthesis becomes more complex under drought stress conditions [97]. Here, GABA regulates plant growth and water use efficiency, which can lead to a dilution effect. In situations of drought stress, GABA application may result in a decreased concentration of essential oils. This illustrates the intricate response of plants to stress factors, where GABA serves as a regulatory agent, subtly adjusting the biochemical changes induced by stress.

Conclusion

The results of the study indicated that water deficiency negatively impacted the growth characteristics of *T. daenensis* Celak. However, the exogenous application of GABA improved the plants' tolerance to drought stress conditions. Applying GABA increased the activity of plant antioxidant enzymes, which helped reduce the harmful effects of reactive oxygen species on plant cells. Additionally, GABA application had positive effects on photosynthetic pigments, such as chlorophylls a and b, which in turn enhanced the productivity of essential oil compounds in thyme seedlings. Overall, the study suggests that the exogenous application of GABA could be recommended to improve plant tolerance to drought stress and to increase essential oil productivity in *T. daenensis* Celak seedlings.

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Statement of compliance
The authors confirm that all the experimental research studies on plants, including the collection of plant material, complied with relevant institutional, national, and international guidelines and legislation.

Statement on experimental research and field studies on plants
The growing plants sampled comply with relevant institutional, national, and international guidelines and domestic legislation of Iran.

Clinical trial number
Not applicable.

Authors' contributions

E.N. and F.S.: Conceptualization, Methodology, Software, Validation, Visualization, Writing- Original Draft, A.A: Formal Analysis, Investigation, Resources, Data curation. MR.M.: Supervision, Project administration, Software, Writing—Original Draft and Revision. M.G.: Writing—Original Draft and Revision. All authors have read and agreed to the published version of the manuscript.

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The raw data of this article will be made available by corresponding author according to the personal requests.

Declarations

Ethics approval and consent to participate

All methods performed in this study including the collection of plant materials were in compliance with the relevant institutional, national, and international guidelines and legislation.

Consent for publication

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Competing interests

The authors declare no competing interests.

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